REMARKS

Formal Matters

Claims 1-3 and 20 are pending.

Claims 1-3 and 20 were examined and rejected.

Claims 1 and 2 are amended. The amendments were made solely in the interest of expediting prosecution, and are not to be construed as an acquiescence to any objection or rejection of any claim. No new matter is added.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Interview Summary

Examiner Ponnaluri is thanked for the telephonic interview held on November 29, 2005, with the Applicants' representatives James Keddie, Carol Francis and Jim Diehl.

Outstanding rejections and arguments to overcome those rejections were discussed.

During the interview, Examiner Ponnaluri agreed that the Applicants' results were surprising in view of the statue of the art at the time of filing, which art would lead the ordinarily skilled artisan to expect that there would not be success in use of a retroviral vector to express a wild-type *Renilla* GFP. The Applicants agreed to re-frame their arguments to emphasize their unexpected results.

Rejections under 35 U.S.C. § 103- general discussion

As a preliminary matter, the Applicants note that the claims are amended to delete the phrase "adapted for transfecting a mammalian cell".

As discussed in the aforementioned interview, retroviral vectors are inherently capable of transfecting a mammalian cell. The Applicants submit that the phrase "adapted for transfecting a mammalian cell" need not be recited in the instant claims.

The rejected claims relate to a retroviral vector containing a polynucleotide encoding wild-type *Renilla* GFP. The Applicants note that the claimed vector is a ***retroviral*** vector and the *Renilla* GFP sequence is ***wild-type***. SEQ ID NO:2 is the amino acid sequence of the wild-type *Renilla* GFP.

The claims stand rejected over Bryan (disclosing a sequence of a wild type *Renilla* GFP) in view of Aran and/or Zolutukhin (each disclosing a retroviral vector encoding human codon optimized, mutated *Aequoria* GFP). According to the Office, Aran and/or Zolutukhin's retroviral vector, in combination with Brian's wild type *Renilla* GFP sequence, render the claims unpatentable.

The claimed retroviral vector may be used to express wild type *Renilla* GFP in mammalian cells. Results supporting this statement are shown in the instant specification.

Simply put, the results achieved with the claimed invention were unexpected because the Applicants found success in an area in which others had found only failure: namely expression of wild type green fluorescent proteins using a retroviral vector. The Applicants' success was surprising because the art at the time of filing shows that wild-type GFPs other than the wild type *Renilla* GFP (i.e., the wild-type versions of the particular mutant GFP used by Aran, Zolutukhin and others) could **not** be expressed in a mammalian cell using a retroviral vector.

The Applicants' position is factually supported by the publications of Aran, Hanazano, Levy, Cheng and, and others, who unsuccessfully tried to express wild-type *Aequoria* GFP using a retroviral vector. The publications were published before the time of filing of the instant application and were readily available at the time of filing.

In support of the Applicants' position, the Examiner's attention is drawn to the first full paragraph of page 204 of Aran's disclosure. In this paragraph, Aran states that when a retroviral vector encoding a wild type *Aequorea* GFP was introduced into in a mammalian cell, fluorescence was "***undetectable***".

Several other research groups -- namely those of Hanazono, Levy, and Cheng -- independently experienced problems in this same effort.

Hanazono (Hum. Gene Ther. 1997, 8:1313-9; Exhibit A) stated in the abstract that "many attempts by our laboratory to isolate stable retroviral producer cell clones secreting biologically active vectors containing either the highly fluorescent S65T-GFP mutant or humanized GFP have ***failed***", and

with reference to retroviral vectors encoding GFP, stated in the overview "***stable clones produced neither virus nor GFP***" and "GFP may not be a suitable selective marker in mammalian gene transfer systems".

Levy et al. (Nature Biotechnology 1996, 14: 610-4, at p. 613, first full paragraph; Exhibit B) states that "Our experiments are in agreement with these results in that transient transfection which transfers multiple transgene copies of wildtype GFP expression cassettes were visualized, but we found that stable transduced lines with a single transgene copy of ***wildtype GFP could never be visualized*** by fluorescence microscopy (Table 1)".

Likewise Cheng et al (Nature Biotechnology 1996, 14: 606-609; paper enclosed as Exhibit C) states in the second paragraph of the introduction "the expression and detection of wildtype GFP (wtGFP) in mammalian cells reportedly ***failed***".

Further, several other publications generally state that wildtype *Aequorea* green fluorescent proteins are toxic to living cells (see Lie et al Biochem. Biophys. Res. Commun. 1999, 260:712-717, Duisit et al. Mol. Ther. 2002 6:446-454 and various publications by Stratagene, enclosed as Exhibits D, E and F).

It was not until the publications of Levy et al (Nature Biotechnology 1996, 14: 610-4; Exhibit B) and Cheng et al (Nature Biotechnology 1996, 14: 606-609; Exhibit C) that red-shifted, humanized, optimized ***variants*** of *Aequorea* GFP suitable for use in retroviral vectors became available. Identifying these *Aequorea* GFP variants was not a trivial task.

Aran, Hanzano, Levi and Cheng clearly state their failure to express wild type *Aequoria* GFP using a retroviral vector.

The Applicants submit that in view of the known difficulties in using retroviral vectors to express the *Aequorea* wild type green fluorescent proteins, one of skill in the art would fully expect that a retroviral vector encoding a wild type *Renilla* GFP would also fail. In direct contrast to the prevailing wisdom at the time of filing, the inventors discovered that ***wild type Renilla GFP*** could, in fact, be expressed in mammalian cells using a retroviral vector. The Applicants submit that this result was unexpected, and could not have been predicted from the art at the time of filing.

During the interview, the Examiner asked the Applicants if they knew of any reason why, in view of the precedent for failure, the inventors found success in using the claimed invention.

It is the Applicants' understanding that exact mechanism by which *Aequoria* GFPs fail to be expressed using a retroviral vector is not known and, as such, the underlying reasons for the Applicants' success are not understood. Furthermore, the Examiner is reminded that it is well established that an understanding of the scientific theory or principle underlying an invention is not a requirement for patentability.[1] Thus, while the exact molecular mechanism underlying the Applicants' success may be an interesting topic for discussion, such a discussion should have no bearing on the patentability of the rejected claims.

The general discussion set forth above supports the Applicants' position that Aran, Bryan and/or Zolutukhin, cannot be combined to render the claimed invention obvious. Withdrawal of rejections that rely on the disclosures of these references is respectfully requested.

Each of the rejections set out in the Office Action is addressed in detail below.

Rejection under 35 U.S.C. § 103 - Bryan and Aran

Claims 1-3 and 20 remain rejected under 35 U.S.C. § 103(a) as being unpatentable over Bryan and Aran. The Office Asserts that Bryan's GFP, in combination with Aran's retroviral vectors, renders the subject matter of the instant claims obvious.

In view of the generally discussion set forth above, the Applicants submit that this rejection has been adequately addressed. Withdrawal of this rejection is respectfully requested.

[1] See, e.g., In re Gazave, 379 F.2d 973, 978, 154 USPQ 92, 96 (CCPA 1967); In re Chilowsky, 229 F.2d 457, 462, 108 USPQ 321, 325 (CCPA 1956) and Philip Morris, Inc. v. Brown & Williamson Tobacco Corp., 641 F. Supp. 1438, 1483 n.13, 231 USPQ 321, 355 n.13 (M.D. Ga. 1986).

Rejection under 35 U.S.C. § 103 - Aran, Bryan and Zolutukhin

Claim 20 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Aran, Bryan and Zolutukhin. The Office Asserts that Aran's retroviral vectors, Bryan's Renilla GFP and Zolutukhin's human codon optimized GFP renders the subject matter of the instant claims obvious.

In view of the generally discussion set forth above, the Applicants submit that this rejection has been adequately addressed. Withdrawal of this rejection is respectfully requested.

Rejection under 35 U.S.C. § 103 - Zolutukhin and Bryan

Claims 1-3 and 20 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Zolutukhin and Bryan. The Office Asserts that Zolutukhin's human codon optimized GFP retroviral vector, in combination with Bryan's *Renilla* GFP, renders the subject matter of the instant claims obvious.

In view of the generally discussion set forth above, the Applicants submit that this rejection has been adequately addressed. Withdrawal of this rejection is respectfully requested.

CONCLUSION

The Applicants submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number RIGL-011.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: December 5, 2005

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EXHIBIT A

HUMAN GENE THERAPY 8:1313–1319 (July 20, 1997)
Mary Ann Liebert, Inc.

Green Fluorescent Protein Retroviral Vectors: Low Titer and High Recombination Frequency Suggest a Selective Disadvantage

YUTAKA HANAZONO, JIAN-MEI YU, CYNTHIA E. DUNBAR, and ROBERT V.B. EMMONS

ABSTRACT

Green fluorescent protein (GFP) has been used as a reporter molecule for gene expression because it fluoresces green after blue-light excitation. Inclusion of this gene in a vector could allow rapid, nontoxic selection of successfully transduced cells. However, many attempts by our laboratory to isolate stable retroviral producer cell clones secreting biologically active vectors containing either the highly fluorescent S65T-GFP mutant or humanized GFP have failed. Vector plasmids containing various forms of GFP and the neomycin resistance gene were transfected into three different packaging cell lines and fluorescence was observed for several days, but stable clones selected with G418 no longer fluoresced. Using confocal microscopy, the brightest cells were observed to contract and die within a matter of days. RNA slot-blot analysis of retroviral producer supernatants showed no viral production from the GFP plasmid-transfected clones, although all clones derived after transfection with an identical retroviral construct not containing GFP produced virus. Genomic Southern analysis of the GFP-transduced clones showed a much higher probability of rearrangement of the proviral sequences than in the control non-GFP clones. Overall, 18/34 S65T-GFP clones and 17/33 humanized-GFP clones had rearrangements, whereas 2/15 control non-GFP clones had rearrangements. Hence, producer cells expressing high levels of these GFP genes seem to be selected against, with stable clones undergoing major rearrangements or other mutations that both abrogate GFP expression and prevent vector production. These observations indicate that GFP may not be an appropriate reporter gene for gene transfer applications in our vector/packaging system.

OVERVIEW SUMMARY

In this study, we systemically examined the production of retroviral vectors expressing green fluorescent protein (GFP) and report that, despite the production of low levels of vector from bulk populations of fluorescing packaging cells soon after transfection, stable clones produced neither virus nor GFP. A significantly higher frequency of rearrangement of the proviral sequences in the stable clones was found by genomic Southern analysis compared to control producer cell lines. This implies a selective advantage for those clones that rearrange the GFP gene and abrogate its expression. Hence, GFP may not be a suitable selective marker for mammalian gene transfer applications in our vector/packaging system.

INTRODUCTION

GREEN FLUORESCENT PROTEIN (GFP) is derived from the jellyfish *Aequorea victoria*. The GFP cDNA encodes a 238-amino-acid protein with a molecular weight of 27 kD (Prasher *et al.*, 1992). Blue light stimulates the excitation and green fluorescence from a cyclized GFP fluorophore formed by serine-65, tyrosine-66, and glycine-67. Light-stimulated fluorescence does not require co-factors, substrates, or additional gene products, unlike other fluorescent markers. The signal is detectable by both fluorescent microscopy or fluorescence-activated cell sorting (FACS) analysis of live cells (Chalfie *et al.*, 1994). However, the fluorescence of wild-type GFP after visible spectrum excitation is not strong enough for most applications. Therefore, variants of GFP, such as S65T-GFP in which ser-

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ino-65 is altered to threonine, have been developed resulting in a red-shifted excitation peak and four- to six-fold improvement in emission intensity (Heim *et al.*, 1995). Recently, another mutant GFP, humanized S65T-GFP (hGFP), was derived to optimize human-type codon usage, possibly resulting in the improved translation of the gene (Levy *et al.*, 1996; Yolotukhin *et al.*, 1996).

In the field of gene transfer technology, marker genes allowing direct and simple detection of successfully transduced cells would be extremely useful (Kain and Ganguly, 1995). The possibility of immediately selecting for successfully transduced viable cells by simple FACS would be especially welcome in the field of gene transfer to hematopoietic stem and progenitor cells (Dunbar and Emmons, 1994). Gene transfer efficiencies, even with widely used and well-developed murine retroviral vectors, are very low and an ability to select and possibly expand transduced cells could greatly improve the possibility for real therapeutic applications. Detection of marker gene products such as β-galactosidase (β-Gal), luciferase, chloramphenicol acetyltransferase (CAT), or alkaline phosphatase involves either cell fixation, which kills the cells, or antibody-mediated detection, which is time-consuming and can be prone to high background. Drug resistance genes allow positive selection of transduced cells only with days to weeks of growth in selective media, likely changing the characteristics of the target cells through terminal differentiation or other processes. These problems have stimulated investigators to search for a better gene marker system that provides timely, accurate, and nontoxic detection of successful transduction in living cells. One of the candidates is GFP.

We have attempted to isolate stable retroviral producer cell lines packaging a retroviral vector containing the GFP gene and the neomycin resistance (*neo*) gene. In this report, we demonstrate that stable clones producing GFP vectors could not be isolated after drug selection despite initial detection of GFP expression in the packaging cell lines. The G418-resistant clones were found to have rearranged at high frequency, precluding GFP expression and vector production, presumably due to a strong selective advantage for loss of GFP expression.

MATERIALS AND METHODS

Plasmid construction

The plasmid pG1Na (Genetic Therapy Inc., Gaithersburg, MD) served as the backbone for our GFP vectors. For pG1NGFP, a splice donor site was inserted 5′ to the *neo* gene, and a splice acceptor site and the S65T variant of the GFP gene (Clontech, Palo Alto, CA) were introduced 3′ of the *neo* gene such that both genes were driven from the 5′ long terminal repeat (LTR). pG1XGFP was produced by inserting the S65T-GFP gene 5′ of the *neo* gene in pG1Na under the control of the 5′-LTR. An SV40 immediate early promoter was then inserted 5′ of the *neo* sequence to drive this gene (Fig. 1). pG1NhGFP was then constructed by replacing the S65T-GFP gene in pG1NGFP with the humanized form of the S65T-GFP (hGFP) gene (Clontech, Palo Alto, CA).

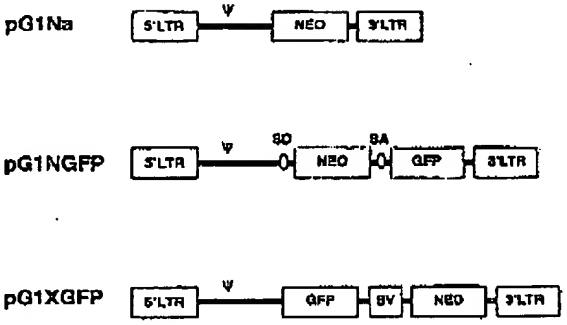

FIG. 1. Retroviral plasmid constructs. The backbone of the retroviral constructs was pG1Na. Two retroviral constructs, both expressing S65T-GFP from the 5′ LTR promoter, were produced. In one construct, a *neo* gene shared the 5′ LTR promoter through a splice donor-acceptor site (pG1NGFP) and in the other construct *neo* was driven by an SV40 promoter independently (pG1XGFP). LTR, long terminal repeat; GFP, S65T form of the GFP gene; NEO, neomycin resistance gene; SV, SV40 immediate early promoter; SD, splice donor site; SA, splice acceptor site; Ψ, viral packaging signal.

Retroviral producer cell lines

Ψ-CRIP (Danos and Mulligan, 1988) and PA317 (Miller and Buttimore, 1986) are murine amphotropic retroviral packaging cell lines. Ψ-2 (Mann *et al.*, 1983) is a murine ecotropic retroviral packaging cell line. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (GIBCO-BRL, Gaithersburg, MD) at 37°C and 5% CO_2. Ψ-CRIP, PA317, and Ψ-2 cells were transfected with plasmids by the lipofectin method according to the manufacturer's protocol (GIBCO-BRL). After 48 hr, some plates were exposed to media containing 400 μg/ml G418 (active) (GIBCO-BRL) and other plates were split 1:5, 1:10, 1:20, 1:50, and 1:100 into the G418-containing selective media. The cells were grown for 10–14 days and either bulk G418-resistant or macroscopic individual clonal populations were expanded for further study. Clones were grown for 2–4 weeks before culture supernatant was harvested for slot-blot hybridization and DNA was extracted for Southern blot analysis.

G1Na.40 is a cloned amphotropic G1Na-producer cell line derived from PA317 (Genetic Therapy Inc., Gaithersburg, MD) with a titer of 1–5 × 10^6 particles/ml.

Shuttle packaging experiments

We used the BOSC23 cells for transient expression of retroviruses (Pear *et al.*, 1993). The cells were transfected with plasmids by the calcium phosphate method described elsewhere (Pear *et al.*, 1993). Two days after transfection, the supernatants containing viruses were collected and filtered. Virus production from the BOSC23 cells was confirmed by the slot-blot analysis, as described below. Packaging cells were infected with the supernatants of the BOSC23 cells as previously described (Migita *et al.*, 1995).

Fluorescent microscopy

A fluorescent confocal microscope with a mercury arc lamp (100 watt) and a fluorescent filter set (consisting of a 480/40-nm excitation filter and a 535/50-nm emission filter) was used to detect GFP in living cells (Nikon, Melville, NY).

Slot-blot analysis

Supernatants from 70–90% of confluent producer cells were filtered through 0.45-μm filters. One milliliter of each supernatant was precipitated with 30% polyethylene glycol 8000 (Fisher Scientific, Fair Lawn, NJ) and the pellets were resuspended with 200 μl of Tris-EDTA buffer (ph 7.4) containing 10% (wt/vol) vanadyl ribonuclease complex (Gibco BRL, Gaithersburg, MD) and 100 μg/ml of yeast tRNA (GIBCO-BRL) and then lysed by adding 200 μl of 2× lysis buffer (1% SDS, 0.6 *M* NaCl, 20 m*M* EDTA, and 20 m*M* Tris-HCl pH 7.4). Retroviral RNA was extracted from the lysed solution by phenol. The RNA was reconstituted with 500 μl of a 7.5% formaldehyde solution containing 1.5 *M* NaCl and 150 m*M* sodium citrate pH 7.0. Next, 100 μl and 400 μl of each RNA sample were loaded into adjacent wells of the slot-blot apparatus Minifold II (Schleicher & Schuell, Keene, NH) with vacuum applied. The transfer membrane (Hybond N^+, Amersham, Cleveland, OH) was hybridized with a *neo* probe or GFP probe generated by PCR. The *neo* gene primers are 5′-ATGATTGAACAAGATGGATTGCA-3′ and 5′-AGGCATCGCCATGGGTCACGACGAGAT-3′. The primer sequences for the S65T-GFP gene are 5′-CTGGA-GTTGTCCCAATTCTTGTTG-3′ and 5′-TCAAGAAGGAC-CATGTGGTCTCTC-3′, and those for the humanized S65T-GFP gene are 5′-TGAACGGCCACAAGTTCAGCGTGT-3′ and 5′-TTACTTG- TACAGCTCGTCCATGCC-3′. Radiolabeling of the probes was done using an oligolabeling kit (Pharmacia, Piscataway, NJ).

Southern blotting

Nuclear DNA was extracted from producer cells or NIH-3T3 cells using a nonorganic DNA extraction kit (Oncor, Gaithersburg, MD). Ten micrograms of DNA was digested with *Sac* I, separated on a 0.8% agarose gel, and transferred onto the nylon membrane Hybond-N^+ (Amersham, Cleveland, OH). The radiolabeled DNA probe was a *neo* or GFP gene-specific sequence as described above.

RESULTS

Preparation of retroviral producer cells

The two retroviral plasmids pG1NGFP and pG1XGFP were modified from the parental plasmid pG1Na (Fig. 1) by the insertion of the S65T form of the GFP cDNA. Amphotropic retroviral packaging cells Ψ-CRIP and PA317 and ecotropic retroviral packaging cells Ψ-2 were transfected with the plasmids pG1Na, pG1NGFP, and pG1XGFP by the lipofectin method. Bulk producers and cloned producer cell lines expressing G1Na, G1NGFP, and G1XGFP were derived after G418 selection as described above in Materials and Methods.

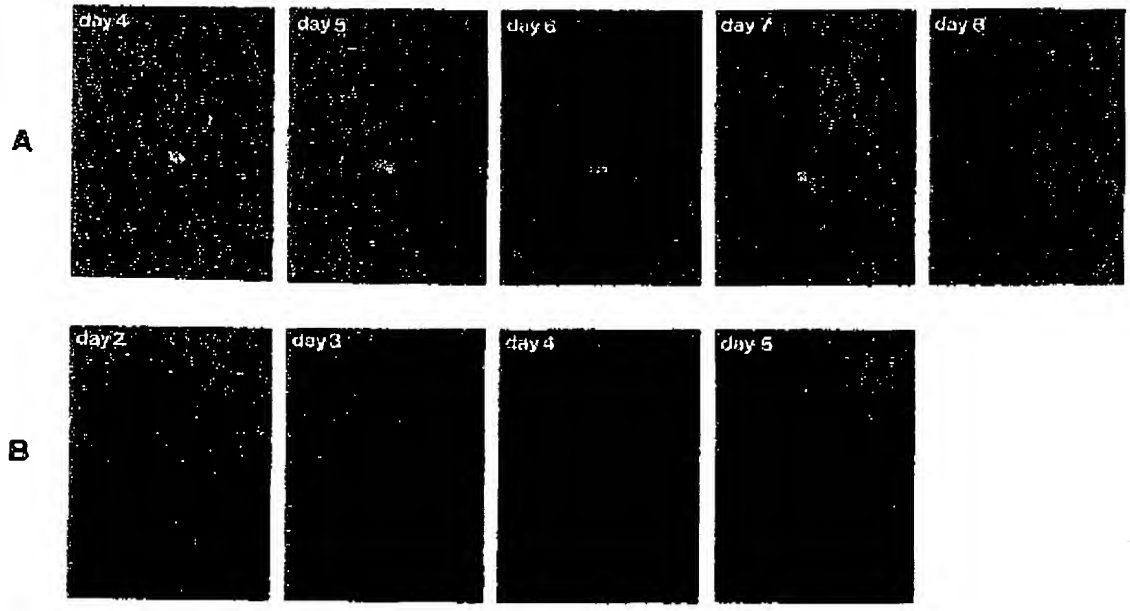

FIG. 2. Fluorescent microscopic observation of the Ψ-CRIP cells transfected with the GFP retroviral plasmid. Ψ-CRIP cells were transfected with pG1NGFP by the lipofectin method. Two days after transfection, the cells were placed in the presence of G418. Two typical examples (A and B) are shown here. Followed under confocal microscopy, the brightest cells were observed to form inclusion bodies and to contract and die within 7–10 days.

Observation of producer cells under fluorescent microscopy

The cells were followed beginning immediately after transfection for fluorescence using confocal microscopy. Fluorescence of cells transfected with the GFP-containing plasmids was observed for below 5% of the cells for the first 3–7 days of growth of the transfected cells, but by 10 days neither stable G418-selected clones nor bulk populations of cells fluoresced. The same loss of fluorescence was observed with or without G418 selection. Followed under confocal microscopy, the brightest cells were observed to contract and die within the first 7–10 days. Figure 2A shows an example of Ψ-CRIP cells transfected with pG1NGFP. A representative, very bright cell is shown at day 4 after transfection. The same cell had developed an inclusion body by day 7, and by day 8 the cell had contracted, died, and no longer fluoresced. Another example shows that two Ψ-CRIP cells transfected with pG1NGFP were fluorescing at day 2 after transfection (Fig. 2B). Some inclusion bodies were detected by day 4, and by day 5 the cells had died and no longer fluoresced. The same pattern of early fluorescence, inclusion body formation, and death of bright cells was seen with PA317, Ψ-2, and 3T3 cell lines transfected with the plasmids under the same conditions. No cells transfected with the control pG1Na plasmid ever fluoresced or showed similar patterns of inclusion body formation and cell death.

Virus production

Retroviral RNA was extracted from supernatants of the Ψ-CRIP producers, and slot-blot analysis was performed using a radiolabeled *neo* probe. The supernatants of the bulk G418-selected producers harvested 10–14 days after transfection were shown to contain low levels of viral RNA (Fig. 3A). There was little difference between amounts of viral RNA produced by bulk populations of cells transfected with pG1Na, pG1NGFP, or pG1XGFP. All were orders of magnitude lower than supernatant from the G1Na.40 clonal producer cell line used as a positive control.

The supernatants from individual producer cell clones selected by growth in G418 and harvested 4–6 weeks after transfection with the pG1Na plasmid were also shown to make viral RNA, although at levels 1–2 logs lower than the positive control producer G1Na.40 (Fig. 3B). However, no evidence of virus production from the pG1NGFP- or pG1XGFP-transfected clones could be obtained by slot-blot analysis (Fig. 3C). A total of 32 clones (9 clones for G1NGFP and 23 clones for G1XGFP) assayed had no detectable viral RNA production. We have repeated the slot-blot analyses looking at virus production from these clones with a GFP probe, and confirmed that no viral particles containing the GFP sequences were being produced (data not shown). We have also confirmed lack of virus production by these producer clones in a functional titering assay: 3T3 cells exposed to the viral supernatants from the G1NGFP or G1XGFP clones in the presence of protamine did not become G418-resistant, and did not show any fluorescence (data not shown).

Infection with transient GFP retroviruses

The BOSC23 cells were also used to test the retroviral constructs and to produce ecotropic retroviruses. We transfected

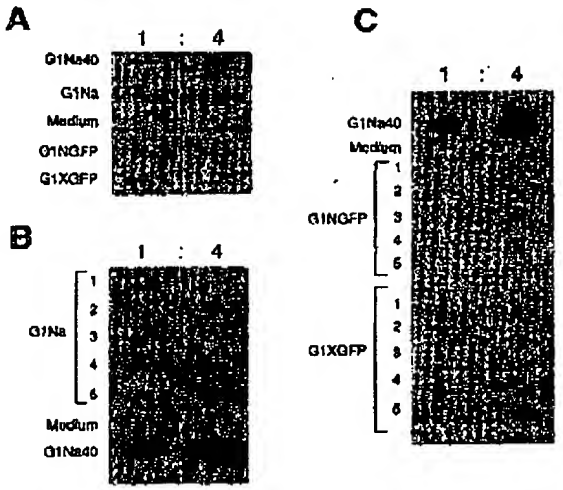

FIG. 3. Slot-blot analysis. Viral RNAs were extracted from 1 ml supernatants of producer cell line conditioned media. The RNAs were loaded into two adjacent wells in the ratio of 1:4 and transferred onto nylon membranes. The membranes were hybridized with a radiolabeled probe of the *neo* gene-specific DNA sequences. The positive control was the cloned producer cell line G1Na.40 supernatant (titer 1–5 × 10^6 particles/ml) and the negative control was the medium. The bulk producers (at 10–14 days after transfection) were shown to produce retroviruses of G1Na, G1NGFP, and G1XGFP (A). However, producer cell clones at 4–6 weeks after transfection for G1NGFP and G1XGFP and selection with G418 no longer produced viral RNA (C), although producer cell clones isolated at the same time containing G1Na still produced viral RNA (B).

the BOSC23 cells with our GFP plasmids by the calcium phosphate method. Two days after transfection, when approximately 80% of the cells fluoresced, the supernatants were harvested and filtered. RNA slot-blot probed with *neo* or GFP sequences revealed a high level of production of viral RNA, higher than a comparable producer cell line (G1Na.40) with a known biologic titer of 10^6 particles/ml (data not shown). The high percentage of cells visibly expressing high-level GFP was most likely due to the multiple copies per cell in the BOSC transiently expressing retroviral producer system. We then infected PA317 and Ψ-CRIP packaging cells with the supernatants. The number of the cells fluorescing was below 1%, probably reflecting the fact that most cells only contained a single copy of the vector, or a potential selective advantage favoring rearrangement of the vector in the transfected BOSC cells, with resultant poor transduction efficiency. The brightest cells infected with the virus died approximately 1 week after infection, even without G418 selection, in the same way as the packaging cells directly transfected with the GFP-containing plasmids.

Southern blot analysis of the producer cell lines

To try and explain the inability of the stable G418-resistant producer clones transfected with the GFP constructs to make retrovirus, Southern blotting was performed. Genomic DNA extracted from the Ψ-CRIP producer cell lines was digested with

Sac I, a restriction enzyme that cuts in the LTRs but not within the viral construct sequence. Each insertion of the plasmid sequences in the producer cell genome should result in 2.4-, 3.4-, and 3.6-kb bands for G1Na, G1NGFP, and G1XGFP, respectively, when probed with an internal probe such as *neo* gene sequences.

There were rearranged bands in four of the five G1NGFP clones studied and in four of five G1XGFP clones (Fig. 4A). Some clones were completely missing the band of the correct size (two of the five clones for G1NGFP, one of the five clones for G1XGFT). One clone of G1NGFP (clone 3) had a very high copy number of the inserted sequence in its genome, presumably due to duplication of the gene. We have reprobed the membrane with a GFP probe: Some bands disappeared, suggesting complete deletion of the GFP sequences, whereas other bands had the same pattern as with the *neo* gene probe, suggesting rearrangements in the viral regions (data not shown).

In the G1Na-producer cell clones derived at the same time, there was a significantly lower frequency of clones with rearranged bands of the incorrect size (Fig. 4B) as compared to either GFP construct. One clone had a larger rearranged band and was missing the correct band (clone 4). Nonetheless, the clone still produced viral RNA (Fig. 3C), indicating that the critical control and packaging sequences were intact. In total, there were 15 rearranged bands in the 10 GFP-producer cell lines, but at most two rearranged bands in the five G1Na-producer cell lines. We obtained similar results with Southern blotting of DNA from the Ψ-2 and PA317 clones (Table 1).

Producer cell lines expressing the humanized GFP

We have constructed pG1NhGFP in which the S65T-GFP gene was replaced by the humanized S65T-GFP (hGFP) gene, transfected the plasmid into Ψ-CRIP, Ψ-2, and PA317 cells, and established the producer cell lines in the same way as above. Early fluorescence, inclusion body formation, death of bright cells, and loss of fluorescence in the cloned producer cells were observed, similar to results with the other GFP genes. The Southern blotting of the humanized GFP-producer clones showed a high frequency of clones with rearrangements (17/33) comparable to that of the native GFP-producer clones (Table 1).

DISCUSSION

Other investigators have recently reported the use of wild-type GFP and more highly fluorescent variants as a genetic marker in retroviral vectors. In one study, retroviruses containing the GFP gene were obtained by transfections of BOSC23 cells (Cheng *et al.*, 1996) and only transient production of retroviruses containing GFP was documented. Another group has established stable producer cells (PA317) expressing the humanized GFP (Levy *et al.*, 1996). However, these producer cells were bulk producers followed for brief periods, not cloned cell lines that would typically be necessary for any preclinical or clinical applications.

We have attempted to isolate cloned stable producer cell lines producing high titer retroviral vectors containing the GFP gene. However, transfected packaging cells with high levels of GFP expression died within several days to weeks, as demonstrated by following individual cells via confocal microscopy. It is known that GFP can form inclusion bodies in *Escherichia coli* when it is expressed at high levels (Cramieri *et al.*, 1996). We observed inclusion body formation prior to cell death in the transfected producer cells. Bulk-selected cells produced low-titer virus for short periods, but cloned cell lines were no longer fluorescent and no longer produced detectable vector RNA or biologically active viral particles. To exclude the possibility that direct transfection of the producer clones prevented successful vector production and stable fluorescence, the BOSC23 system

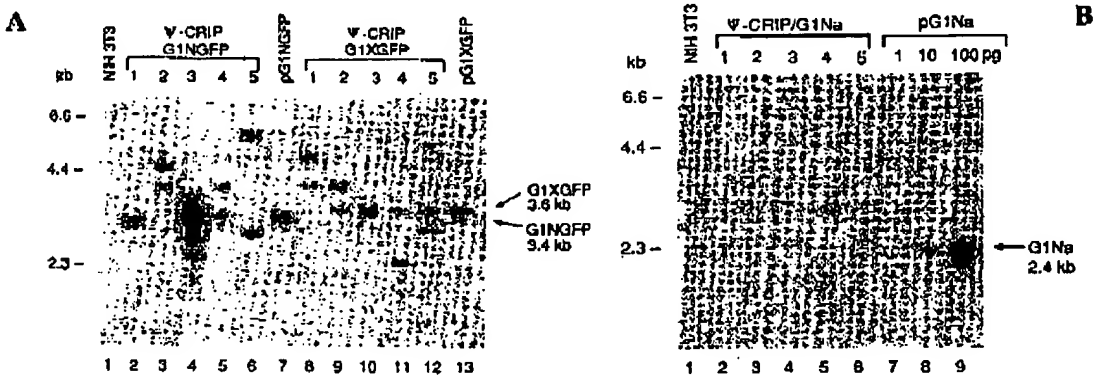

FIG. 4. Genomic Southern blotting of producer cell clones. GFP- (A) and G1Na-producer cell lines (B). Genomic DNAs (10 μg of DNA per lane) were extracted and treated with *Sac* I, which cuts in the LTRs but not within the viral construct sequences. The membranes were hybridized with a radiolabeled probe of the *neo* gene-specific DNA sequences. The plasmids, pG1NGFP (10 pg of the plasmid, lane 7 in A), pG1XGFP (10 pg of the plasmid, lane 13 in A), and pG1Na (1 pg of the plasmid, lane 7; 10 pg, lane 8; 100 pg, lane 9 in B) were used as the positive controls. Ten picograms of pG1NGFP and pG1XGFP corresponds to 1.6 copy per cell, and 10 pg of pG1Na corresponds to 2.5 copy per cell. Genomic DNA from NIH-3T3 cells was used as the negative control (lane 1 in A and lane 1 in B).

TABLE 1. REARRANGEMENT FREQUENCIES

	G1Na			G1NGFP			G1XGFP			G1NhGFP		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Ψ-CRIP	5	2	2	5	4	9	5	4	6	11	8	26
PA317	6	0	0	6	1	3	6	2	3	11	3	4
Ψ-2	4	0	0	6	4	5	6	3	3	11	6	10
Total	15	2	2	17	9[d]	17	17	9[e]	12	33	17[f]	40

a, number of clones examined; *b*, number of clones with rearranged bands; *c*, total number of rearranged bands.
[d]$p = 0.017$ (9/17 *vs.* 2/15).
[e]$p = 0.017$ (9/17 *vs.* 2/15).
[f]$p = 0.011$ (17/33 *vs.* 2/15).

was used to infect the same packaging cell lines, with similar loss of fluorescence and death of initially bright producer cells.

Clones that had rearranged the proviral genome in such a way that GFP expression was abrogated but that retained the *neo* gene necessary for selection appeared to have a strong advantage. This phenomenon was not seen in clones derived from transfection of packaging cells with non-GFP-containing vector sequences. The GFP-containing clones not shown to have gross DNA rearrangements of the provirus still did not produce viral particles. Presumably smaller-scale deletions, rearrangements, or even point mutations occurred in those cell lines that were not detectable by Southern blot analysis, but that nonetheless abrogated both GFP expression and virus production without abrogation of the *neo* gene expression.

The same problems were observed even when using humanized GFP, which was derived to take advantage of optimized human-type codon usage in hopes of improving translation (Levy *et al.*, 1996; Zolotukhin *et al.*, 1996). The base pair changes are all silent, thus the native and humanized GFP genes differ only in their nucleic acid sequences and have identical amino acid sequences. This suggests that the problem of selective disadvantage seems to be associated with expression of the GFP protein, not the gene itself or RNA expression.

It is less likely that the vector backbone itself becomes inherently unstable when expressing two transgenes. We have succeeded in establishing several producer cell lines containing the *neo* gene in the same position and expressing a second gene inserted into exactly the same cloning site as GFP, including G1NaIL3 (murine interleukin-3) and G1XmIFNγ (murine interferon-γ). Clones producing slot-blot-detectable, biologically active virus were obtained at high frequencies after G418 selection: 17/17 for G1NaIL3 and 32/40 for G1XmIFNγ (data not shown).

We have also transfected NIH-3T3, COS, and HeLa cells with non-retroviral plasmids containing S65T-GFP to establish stable cell lines expressing GFP. Despite initially fluorescent populations of cells, over time the cells were no longer fluorescent and similar cell death phenomena and inclusion bodies were observed under confocal microscopy. Thus, this phenomenon was not restricted to producer cells or to proviral plasmids. Transfected HeLa cells retained fluorescence for the longest period (2–3 weeks), possibly because HeLa cells are highly transformed and may be more resistant to the deleterious effects of GFP. There is one report of stable mammalian cell lines (BHK and CHO cells) expressing GFP (Olson *et al.*, 1995). But GFP was expressed as part of a fusion gene product with a cytoskeletal protein. This may have targeted the GFP to a location within the cell that was less problematic. Transgenic mice expressing the GFP gene have been reported (Ikawa *et al.*, 1995), but a systematic examination for any toxicity of the gene product has not been performed. Problems with GFP have been reported in other model systems. In plant cell transfections, it has been difficult to regenerate fertile plants from the brightest transfectants (Haseloff and Amos, 1995).

The lack of virus production by cell lines containing specific vector sequences is not limited to constructs containing the GFP gene. Similar phenomena have been observed in producer cell lines expressing oncogenes, including *abl* and *rel* (Pear *et al.*, 1993). Initially, the cell lines were shown to produce retroviruses at relatively high titers, but titers fell sharply with continued propagation of the cell lines. Although mechanisms were not well understood, it was hypothesized that it was due to some deleterious effect of the gene product (Ziegler *et al.*, 1981).

Recently, another form of mutant GFP was generated by using DNA shuffling to produce molecular evolution, with resulting 42-fold improvement in fluorescence over wild-type GFP (Crameri *et al.*, 1996). This new variant form of GFP may change the behaviors we have observed. Another possible approach would be to use an inducible promoter, allowing transient expression of GFP for a long-enough time to allow selection of transduced cells but perhaps brief enough to avoid significant toxicity. Currently, GFP is not suitable for inclusion as a selectable marker in the well-characterized vector/packaging systems utilized in this paper, but further understanding and modification of this unique protein may allow more successful applications.

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EXHIBIT B

RESEARCH ARTICLE

Retroviral transfer and expression of a humanized, red-shifted green fluorescent protein gene into human tumor cells

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Over two-thirds of the current gene therapy protocols use retroviral gene transfer systems. We have developed an efficient retroviral-based method that allows rapid identification of gene transfer in living mammalian cells. Cells were generated containing a gene for an improved (humanized, red-shifted) version of the *Aequorea victoria* green fluorescent protein (hRGFP) from a retroviral vector. The hRGFP gene was used to produce an amphotropic vector producer cell line that demonstrated vibrant green fluorescence after excitation with blue light. A375 melanoma cells transduced with the retroviral vector demonstrated stable green fluorescence. Both PA317 murine fibroblasts and A375 human cell lines containing the vector were easily detected by FACS analysis. These vectors represent a substantial improvement over currently available gene transfer marking systems. Bright, long-term expression of the hRGFP gene in living eukaryotic cells will advance the study of gene transfer, gene expression, and gene product function in vitro and in vivo particularly for human gene therapy applications.

Keywords: retrovirus, green fluorescent protein, gene transfer, vector producer cell

Since the inception of DNA transfer technology, there has been an intense interest in gene marker systems that allow direct observation of transferred genes into living cells. Murine retroviral vectors have emerged in the past several years as the most common vehicle to deliver marker genes. Detection of markers such as β-galactosidase, luciferase, chloramphenicol acetyltransferase, and alkaline phosphatase involves either cell fixation that kills the cells or antibody-mediated detection. These methods are often time consuming and are prone to endogenous high background. Another group of gene transfer markers convey drug resistance and thus allow positive selection of transfected cells through selection of resistant colonies. Although drug selectable markers allow the detection of living cells expressing the transgene, they require that the cells survive in a toxic environment over a long period of time. These problems have led investigators to search for a better gene marker system that provides timely and accurate detection in living cells. One of the most promising new markers being developed to investigate gene transfer is the green fluorescent protein (GFP).

A number of species use a bioluminescent GFP to emit green light after energy transfer from either luciferases or photoproteins[1]. The GFP gene product can function as a marker in living cells and animals and does not require a substrate (other than light) to visualize gene transfer[2]. The GFP gene was cloned from the jellyfish *Aequorea victoria*, and the protein was found to have extremely stable fluorescence in vitro after stimulation with blue light[3].

The GFP cDNA encodes a 238 amino acid polypeptide with molecular weight of 27 kD[3]. GFP acts as an energy-transfer acceptor that under physiologic conditions in *A. victoria* receives energy from an activated aequorin-coelenterazine complex[4,5]. The chromophore is produced from autocyclization of three residues, serine-65, dehydrotyrosine-66, and glycine-67[4,6]. GFP is inactive until cyclization and oxidation of these three residues to generate a *p*-hydroxybenzylideneimadazolidinone chromophore (Fig. 1). Molecular oxygen is required for fluorophore formation[7]. The formed protein can be column purified, renatured, and crystallized and still maintain its fluorescent characteristics[8,10]. These results prompted expression studies of wildtype GFP in prokaryotic and eukaryotic cells[2]. This basic understanding of GFP mechanistic properties has led, in turn, to additional modifications to extend its usefulness to other systems. Recently, a gain of function mutant GFP gene was generated by Heim and colleagues[11] that altered the serine-65 codon to a threonine codon resulting in a red-shifted excitation peak. This red shifted GFP demonstrated superior fluorescence characteristics compared to wildtype GFP in prokaryotes[11].

A few investigators have humanized the wildtype codons[12]. *A. victoria* is classified in the phylum Ctenophora, and its codon usage is significantly different from that of mammals. Due to these differences, mammalian cells may not efficiently translate wildtype GFP mRNA. In this report, we describe the cloning and characterization of amphotropic retroviral vectors capable of demonstrating efficient, stable transfer of a humanized, red-shifted GFP (hRGFP) gene into mammalian cells. Retroviral vector-transduced living cells have a stable, bright green fluorescence after excitation with blue light. PA317 VPC or A375 melanoma cells expressing the hRGFP vector demonstrated an intense greenfluorescence by FACS analysis. These findings pave the way for a wide variety of experimental and clinical applications of modified GFP genes.

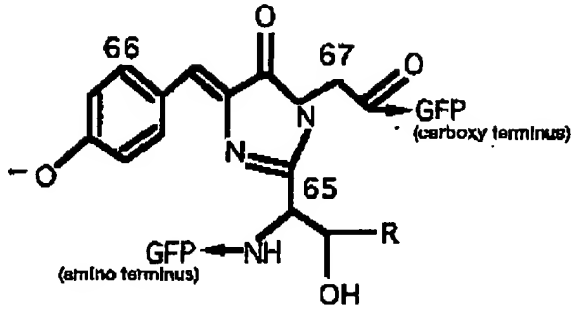


Figure 1. The GFP fluorophore. Blue light stimulates the excitation of a cyclized wildtype GFP fluorophore formed by serine-65, tyrosine-66, and glycine-67, emitting green fluorescence after stimulation. The red-shifted variant contains a mutation that converts serine-65 to threonine. This results in a red shift of the excitation wavelength, increased amplitude of fluorescence, and a faster rate of fluorophore formation in the mutant GFP[11]. R = H (serine); R = CH3 (threonine).

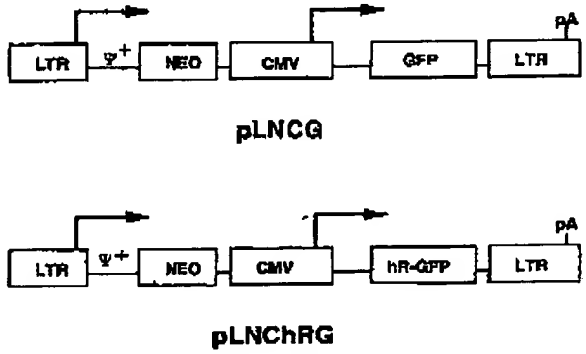


Figure 2. Retroviral constructs containing the red-shifted, humanized green fluorescent protein. The wildtype GFP and the humanized, red shifted GFP gene were cloned into the pLNCX retroviral backbone[11]. Plasmid pLNCG was constructed by subcloning a wildtype GFP DNA fragment into pLNCX. The LNChRG vector was constructed by subcloning a fragment containing the humanized, red-shifted GFP coding region into the multi-cloning site of pLNCX. (See Experimental protocol). LTR, long terminal repeat; pA, polyadenylation signal; arrows indicate transcriptional start sites; 'Ψ' indicates the presence of the viral packaging sequence; GFP, wildtype green fluorescent protein; hRGFP, humanized, red-shifted GFP.

Results

Transfected cell lines. PA317 retroviral packaging cells and A375 melanoma cells were transiently transfected with pLNCG or pLNChRG plasmids (Fig. 2). These two constructs led to significantly different levels of fluorescence after transient transfection (Table 1). Nontransfected PA317 cells did not demonstrate green fluorescence (Fig. 3A). Transfected PA317 cells containing the wildtype GFP gene (pLNCG) exhibited fluorescence in <2% of the cell population that was detected after 48 h (Fig. 3B). However, transfection results with the humanized, red-shifted GFP retroviral construct (pLNChRG) were outstanding. Fluorescence can be detected as early as 9 h posttransfection. By 36 h, 30% to 40% or more of the cells are easily visualized, and contain enough protein to produce an intense fluorescence (Fig. 3D). Overall, with the fluorescence detection filters used, pLNChRG transfected PA317 cells had enhanced fluorescent intensity and efficiency compared to PA317 cells transfected with wildtype GFP plasmid (pLNCG). We did not observe any cytopathic or growth-inhibiting effect due to GFP or hRGFP in transfected cells.

Stable LNCG and LNChRG retroviral vector producer cells. Stable LNCG or LNChRG PA317 VPC were generated by lipofection. Transfected PA317 cells were selected in media containing G418 (1 mg/ml). These stable LNCG or LNChRG PA317 VPC were examined by fluorescence microscopy. The LNCG PA317 VPC line that contains the wildtype GFP gene exhibited no visible fluorescence after excitation with 420–470 nm light (data not shown). We therefore analyzed the LNCG VPC line by PCR using GFP amplimers to detect host chromosomal integration of the LNCG vector. The GFP gene was present in all lines tested (data not shown). In striking contrast, the LNChRG PA317 VPC line demonstrated vibrant green fluorescence in nearly 100% of the cells after G418 selection (Fig. 4). The fluorescence in the LNChRG VPC line was capable of highlighting many subcellular organelles.

Detection in LNChRG transduced A375 melanoma and NIH3T3tk- transduced cell lines. Supernatants from cultures of LNCG or LNChRG PA317 VPC were collected when the cells were 90% to 100% confluent. Supernatants were filtered and transferred to tissue culture plates containing A375 melanoma cells or NIH3T3tk- cells. Twenty-four hours after the final exposure to the retroviral supernatants, the target cells were placed under G418 selection. Most cells will contain only one integrated copy of the retroviral vector (C. Link, unpublished results). A375 cells transduced by LNCG VPC demonstrated no evidence of fluorescence despite the fact that PCR revealed the presence of GFP in the cellular genome (data not shown). However, the *neo*^{r} gene transferred by the LNCG vector was functional, since the A375 cell colonies were G418 resistant. In contrast, the LNChRG vector transduced A375 melanoma cells had bright fluorescence with nearly 100% of the selected cells demonstrating brilliant green fluorescent activity (Fig. 5A). Similarly, murine NIH3T3tk- fibroblasts transduced with the LNChRG retroviral vector demonstrated strong fluorescence in nearly 100% of the cells (Fig. 5B). A375 and NIH3T3tk- cells, which do not contain a transduced LNChRG, do not exhibit any detectable fluorescence (unpublished results). We did not observe any cytopathic or growth-inhibiting effect due to GFP or

Table 1. Cell lines expressing green fluorescent protein.

Target cell line	GFP gene transferred	GFP gene transfer method	Fluorescence intensity*	%Fluorescent cells@
PA317 fibroblast	GFP	Transient transfection	+	<2%
	GFP	Stable transfection	0	0
	hRGFP	Transient transfection	+++++	30–40%
	hRGFP	Stable transfection	+++	>99%
A375 melanoma	GFP	Transient transfection	+	<2%
	GFP	Stable retroviral transduction	0	0
	hRGFP	Transient transfection	+++	30–40%
	hRGFP	Stable retroviral transduction	+++	>99%

GFP: wildtype GFP gene without red-shift mutation or codon modifications; hRGFP: GFP gene modified to convert codon 65-serine to threonine and codon sequences modified to common mammalian usage. * Relative fluorescence intensity under examination by microscopy. @ Percentage of cells exhibiting fluorescence in the transfected or transduced population.

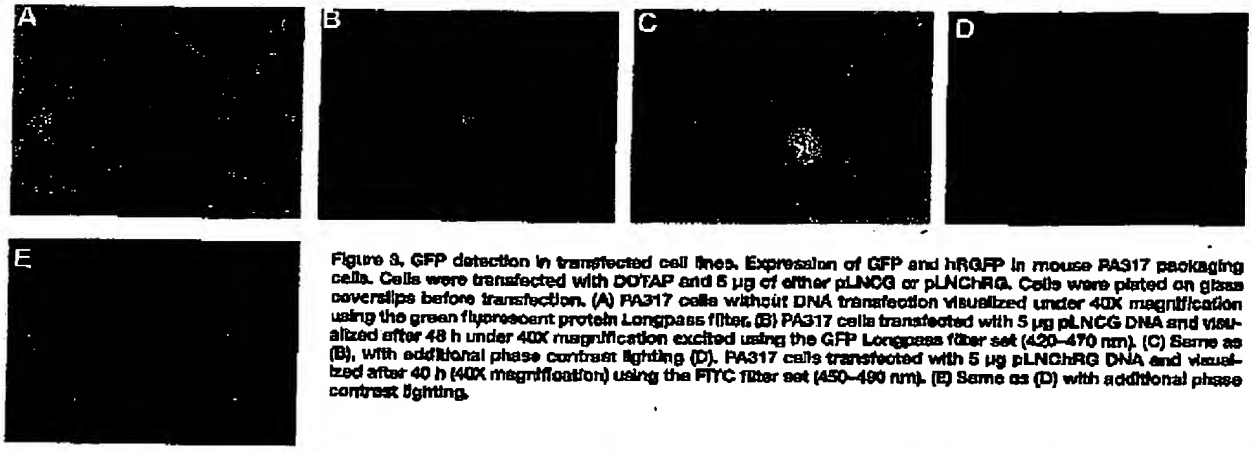


Figure 3. GFP detection in transfected cell lines. Expression of GFP and hRGFP in mouse PA317 packaging cells. Cells were transfected with DOTAP and 5 µg of either pLNCG or pLNChRG. Cells were plated on glass coverslips before transfection. (A) PA317 cells without DNA transfection visualized under 40X magnification using the green fluorescent protein Longpass filter. (B) PA317 cells transfected with 5 µg pLNCG DNA and visualized after 48 h under 40X magnification excited using the GFP Longpass filter set (420–470 nm). (C) Same as (B), with additional phase contrast lighting (D). PA317 cells transfected with 5 µg pLNChRG DNA and visualized after 40 h (40X magnification) using the FITC filter set (450–490 nm). (E) Same as (D) with additional phase contrast lighting.

Figure 4. Detection of hRGFP gene activity in stable, transfected PA317 vector producer cells. Expression hRGFP in mouse PA317 packaging cell after transfection with pLNChRG and selection. Selected cells were plated onto glass coverslips. PA317 cells transfected with pLNChRG were visualized >24 h after replating using the FITC filter set (40X magnification).

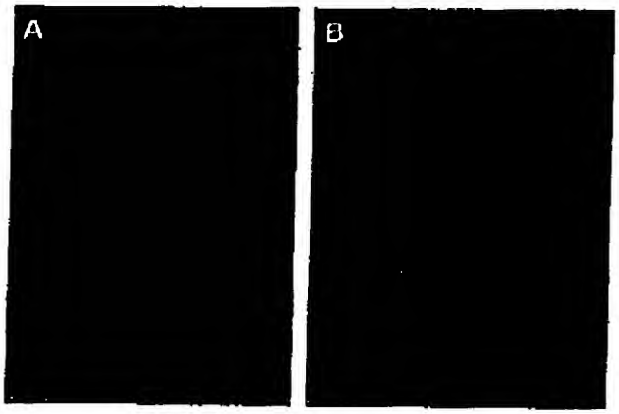


Figure 5. Expression of hRGFP in human A376 melanoma and murine NIH3T3tk- fibroblast cells after transduction with the LNChRG retroviral vector. G418 selected cells were plated onto glass coverslips and visualized using the FITC filter set. (A) A375 melanoma cells transduced with LNChRG retroviral vector (40X) magnification. (B) NIH3T3tk- murine fibroblast cells transduced with LNChRG retroviral vector (40X magnification).

hRGFP in transduced cells.

FACS analysis of GFP transfected PA317 vector producer cells and LNChRG transduced A375 melanoma cells. PA317 cells that had been transfected by the LNChRG vector and selected were analyzed by FACS. Excitation with 488 nm light resulted in light emissions at 525 nm in LNChRG-containing cells. PA317 cells transfected and expressing hRGFP (Fig. 6B) were easily detected by a two-log shift from nontransfected control PA317 cells (Fig. 6A). A375 melanoma cells transduced and selected with the LNChRG vector (Fig. 6D) were readily detected after excitation by shift in detected fluorescence over two logs compared to the control nontransduced A375 cells (Fig. 6C). These results demonstrate that GFP fluorescence can be quantified with available instrumentation.

Discussion

We have demonstrated the effectiveness of a humanized, red-shifted, mutant GFP by retroviral mediated gene transfer into human tumor cells and murine fibroblasts. Mutations of the wildtype GFP gene have resulted in GFP gene products with modified excitation and emission spectra[7,9]. The longer wavelength excitation peak (475 nm) of native *A. victoria* GFP has lower amplitude than its shorter wavelength excitation peak (395 nm)[7]. Heim and colleagues[11] used mutagenesis of the fluorophore to alter the serine-65 residue. They reported gain of function mutants—in which serine-65 was replaced with alanine, leucine, cysteine, or threonine—that show a single excitation peak (470–490 nm) with fluorescence amplitudes from fourfold to sixfold greater than the wildtype gene product. Interestingly, this mutant also shows a more rapid formation of the fluorochrome. We modified GFP such that it contained the red-shifted mutation and codons most commonly translated in mammals[12]. We have evaluated this humanized version of a serine-65 to threonine codon mutant that demonstrates emission at 510 nm in our current gene transfer experiments. Comparisons between the wildtype GFP and the humanized, serine-65 red-shifted mutant (hRGFP) demonstrated substantial improvement in fluorescence expression after either

transfection or retroviral-mediated GFP gene transfer (Table 1).

Wildtype-GFP and GFP fusion proteins have been used in mammalian cells as markers of protein trafficking and gene expression[14–18]. Of note, these experiments used transient transfection to obtain very high levels of wildtype GFP expression. Our experiments are in agreement with these results in that transient transfection which transfers multiple transgene copies of wildtype GFP expression cassettes were easily visualized, but we found that stable transduced cells with a single transgene copy of wildtype GFP could never be visualized by fluorescence microscopy (Table 1). However, our results demonstrate that a humanized, red shifted GFP transgene in single copy can produce excellent fluorescence (Fig. 5). Transgenic mice have been produced expressing a slightly modified wildtype GFP gene expressed from the chicken β-actin promoter[19]. The fingers and tails of these transgenic mice were distinguishable as green under a fluorescent microscope, and homogenized tissue from the muscle, pancreas, lung, and kidney demonstrated fluorescence after excitation with 490 nm light. The visualization of vector gene expression in living transduced tissues with hRGFP may become an outstanding method to study in vivo gene transfer used in human clinical trials.

Experimental protocol

Cell culture. A375 is a human melanoma cell line (ATCC, Rockville, MD). PA317 is a murine amphotropic, retroviral vector packaging cell line (kindly provided by A.D. Miller, University of Washington, Seattle). NIH3T3tk- is a murine fibroblast cell line (kindly provided by Dr. Robert Goldberg, NIH/NCI). Cells were grown in RPMI supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD) in monolayers at 37°C and 5% CO_2. Retroviral vector producer cells (VPC) were grown in RPMI with 10% FCS in monolayers at 37°C and 5% CO_2. All cells were passaged and harvested by standard (Gibco BRL) digestion at 37°C. Cells were routinely passaged at 80% to 90% confluence.

Plasmid preparation and digoxin probes. pGFP-C1 containing wildtype GFP was obtained from Clontech (Palo Alto, CA). pTR-UF2 containing the hRGFP was kindly provided by Dr. Sergei Zolotukhin and Dr. Nicholas Muzyczka (University of Florida, Gainesville). Plasmid DNA was transformed into DH5α competent cells and colonies grown on L-broth supplemented with ampicillin (50 μg/ml) plates (LB/AMP) and transferred onto nylon membranes. The membrane was probed with aDig-GFP probe using a digoxin probe kit (Boehringer-Mannheim, Indianapolis, IN). Primers for the Dig-GFP probe amplification of a GFP fragment were 5' primer 5' GGG AAG CTT TTA TTA TTT GTA TAG TTC ATC CAT GCC and 3' primer 5' GGG AAG CTT GCG CGT ATG GGT AAA GGA GAA GAA CTT. Positive colonies were grown up in LB/AMP broth, and plasmid DNA was isolated using the Qiagen plasmid prep kits (Qiagen, Chatsworth, CA).

Construction of GFP retroviral vectors. Primers were made to amplify the 5' end of the CMV promoter/enhancer and the 3' end of the wildtype GFP gene from the GFP-C1 vector (Clontech). The 5' primer includes unique XbaI, BamHI, and NotI restriction enzyme sites: 5' GGA TCT AGA GGA TCC GCG GCC GCC TAG TTA TTA ATA GTA ATC AAT TAC GGG GTC. The 3' primer includes 3 in-frame stop codons followed by a HindIII restriction enzyme site: 5' GGA AAG CTT CTA TCA TTA TTG AGC TCG AGA TCT GAG TCC GGA CTT GTA. The 1.3 kb CMV-GFP PCR product was cloned into PCR3-cloning vector (Invitrogen, San Diego, CA) to generate plasmid pPCR3CG-14. The 1.3 kb fragment containing the CMV promoter and GFP gene was gel isolated (Jetsorb, Genomed, Bad Oeynhausen, Germany) from the pPCR3CG-14 vector using BamHI and HindIII restriction digest. The 800 bp retroviral CMV promotor was isolated from pLNCX using BamHI and HindIII and discarded. After gel isolation of the remaining 5.8 kb retroviral vector fragment, the 1.3 kb CMV-GFP insert was directionally cloned to generate the pLNCG construct. Finally, plasmid pTR_{UF}-UF2 (Gene Bank Accession # U50963) was restriction digested with NotI and the 730 bp DNA fragment containing the humanized red shift GFP open reading frame was isolated. After Klenow treatment, the blunt ended DNA was ligated into pLNCX at the HpaI site. The resulting plasmid was designated pLNChRG.

Fluorescent detection of green fluorescent protein expressing cells. We visualized GFP expressing cells with a Nikon Labophot-2 Fluorescent Microscope. The cube used in the microscope was either the green fluorescent protein Longpass 41015 filter set (excitation at 420–470 nm and emission at 490 to >600 nm) for the wildtype GFP detection (Chroma Technology Corporation) or the FITC dichromic filter set (excitation at 450–490 nm and emission at 520 nm) for the hRGFP detection. Photographs were taken using the Nikon Microflex UFX-DX and AFX-DX systems.

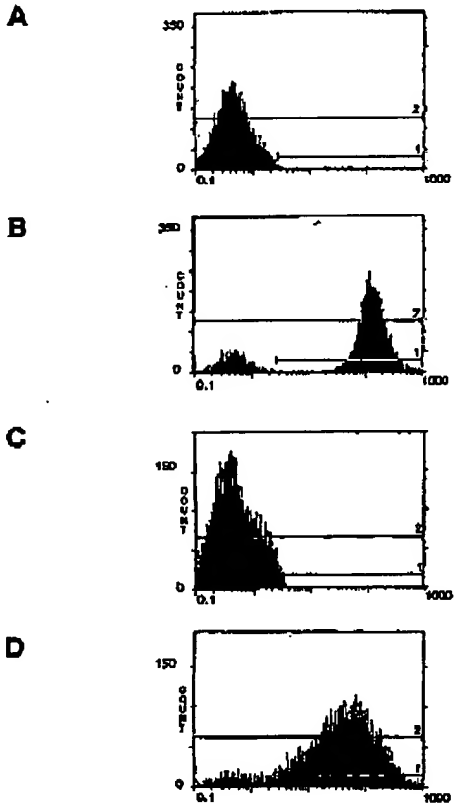
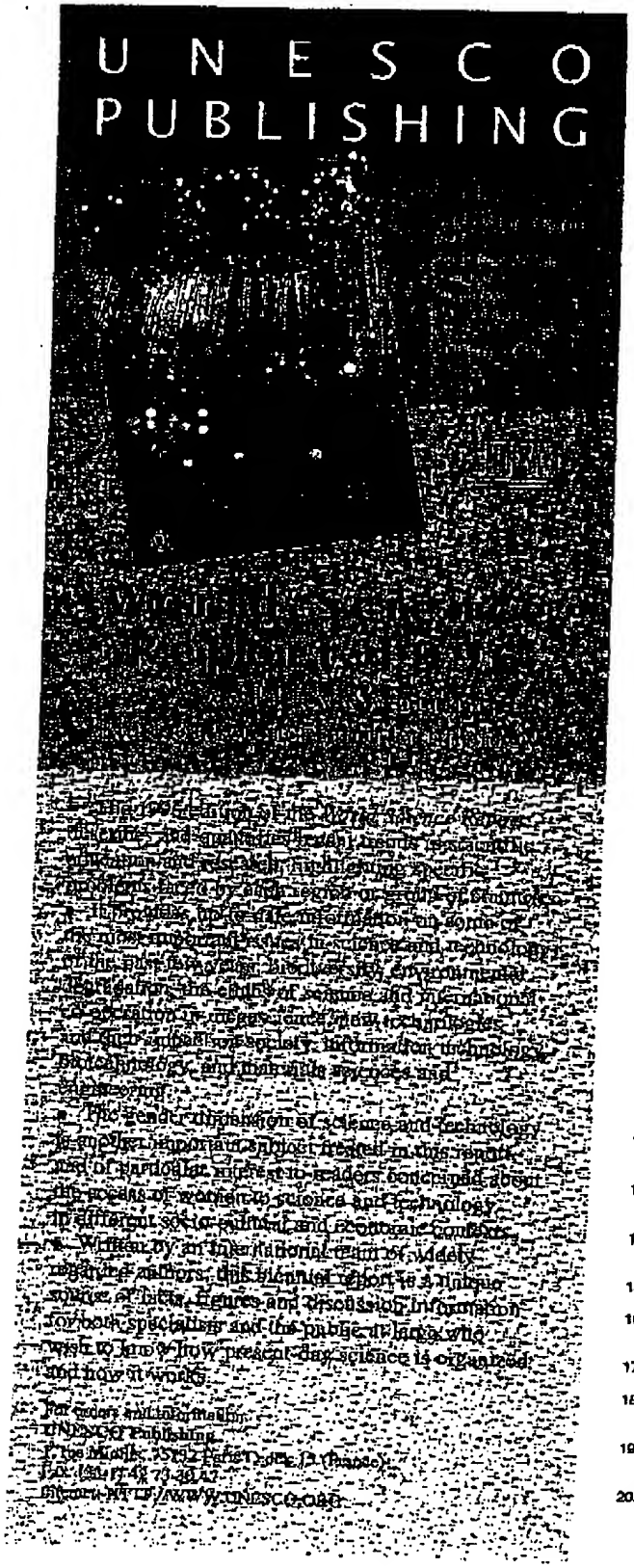


Figure 6. FACS analysis. Determination of hRGFP activity in stable populations of mammalian cells. Cells were trypsin-digested and washed before analysis in a EPICS Profile II Analyzer. (A) PA317 packaging cells without transfection (negative control). (B) LNChRG transfected PA317 vector producer cells expressing hRGFP after excitation. The large shift in peak detected mean fluorescence corresponds with hRGFP activity. (C) Nontransduced A375 cells (negative control). (D) LNChRG transduced A376 cells expressing hRGFP after excitation. The large shift in peak detected mean fluorescence corresponds with hRGFP activity. The FL1 emission channel was used to monitor green fluorescence. Count: cell number counted at given fluorescence intensity; y-axis: a log scale of mean intensity.

Transient expression of GFP. PA317 cells were seeded on a sterilized coverslip in a 6-well dish 12 to 24 h before transfection. Cells were at 30% to 50% confluence at the time of DNA transfection. Five μg of DNA and 15 μl of DOTAP reagent (Boehringer Mannheim) was used as per the manufacturer's protocol. The mixture was added to the plates containing either RPMI 1640 with 10% FBS, 1% L-glutamine, and penicillin/streptomycin or in serum free media. After 10 to 18 h the media was replaced with RPMI with 10% FCS. The coverslip containing the cells was placed on a slide and examined for fluorescence 9 to 48 h after transfection. The cells remaining in the well (after the coverslip was removed) were trypsin digested and transferred to tissue culture dishes. After attachment these cells were placed under selection with G418 (1 mg/ml) for 10 to 14 days.



RESEARCH ARTICLE

Construction, subcloning, and titering of LNCG and LNChRG vector producer cell lines. The plasmid pLNCG or pLNChRG was transfected with DOTAP into the amphotropic retroviral packaging line PA317. Twenty-four hours later, the cells were placed under G418 (1 mg/ml) selection for 10–14 days. LNCG or LNChRG VPC were grown to approximately 90% confluence and supernatants were removed to transduce target cells. Retroviral supernatants were filtered through 0.45 μm filters (Nalgene, Kent, UK), supplemented with 10 μg/ml of protamine sulfate and used to transduce A375 melanoma or NIH3T3tk- fibroblast cells. The target A375 melanoma or NIH3T3tk- cells were 40% to 60% confluent when transduced. Twenty-four hours after the final transduction, cells were placed under G418 (1 mg/ml) selection for 10 to 14 days. Cells were examined by fluorescence microscopy after reseeding the cells onto glass cover slips.

Fluorescence activated cell sorter analysis of transduced human cells. Cytometry of stable hRGFP transfected or transduced cells was performed on a Epics Profile II Analyzer. Cells were analyzed using a 525 nm band pass filter set (Coulter Corp., Miami, FL). Cultures of nontransfected PA317 cells, LNChRG transfected PA317 cells, nontransduced A375 cells, or LNChRG transduced A375 cells that were 80% to 90% confluent were trypsin digested, washed with RPMI with 10% FCS, and resuspended at a concentration of approximately 1×10^{6} cells/ml. All FACS analysis used the FL1 emission channel to monitor green fluorescence (normally a FITC monitor).

Acknowledgments

We thank Dr. Steve Kain and Dr. Paul Kitts (Clontech, Inc.) for helpful discussions. We thank Jeannie Malatesta, Ginger Drolfurst, and Julie Seiwert for technical assistance with flow cytometry. We thank Tom Radoscvich for Internet advice.

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EXHIBIT C

RESEARCH ARTICLE

Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells

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Two mutants of the green fluorescent protein (GFP), RSGFP4 and GFPS65T, have been recently created which differ from the wildtype GFP of *A. victoria* in their excitation maxima. Here we show that human fibroblasts transfected with either of the two mutant GFP genes emit a green fluorescence that is 18-fold brighter than the cells transfected with the wildtype GFP gene. Retroviral vectors expressing the improved GFP gene were also constructed to determine their suitability for stable gene transduction into mammalian cells. The inclusion of the RSGFP4 gene in a retroviral vector did not reduce the viral titer and resulted in a fluorescent signal in viable transduced cells detectable by both fluorescence microscopy and fluorescence-activated cell sorter (FACS) analysis. Therefore, the improved mutant GFP provides a vital marker for monitoring gene transfer and expression in mammalian cells.

Key words: GFP, FACS, fluorescence microscopy, gene transduction, gene therapy

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become an important marker of gene expression[1]. The monomeric GFP consists of 238 amino acids and requires no other *Aequorea* proteins, substrates, or cofactors to fluoresce[1,2]. Thus, it is superior to other reporters such as the *E. coli* lacZ enzyme which requires transport of a fluorogenic substrate across cell membranes, or to cell-surface epitope markers which require specific-antibody staining.

Detection of green fluorescence from the expressed GFP cDNA has been demonstrated in several heterologous systems including *E. coli*, *C. elegans*, and *D. melanogaster*[3-5]. However, the expression and detection of wildtype GFP (wtGFP) in mammalian cells reportedly failed[3]. Recently a fluorescent signal was detected by fluorescence microscopy when GFP-expressing vectors were transfected into cultured cells in which transgenes can be highly expressed[6,7] (unpublished data). Thus, the wtGFP expressed in cultured mammalian cells emits a detectable green fluorescent signal, albeit at a relatively low level.

The recombinant wtGFP purified from *E. coli*, as well as its native form, emits bright green light ($\lambda max = 508$ nm) when excited with an ultraviolet (UV) source ($\lambda max = 395$ nm), or fluoresce weakly when excited with a blue light ($\lambda max = 470$ nm). Excitation with a UV light rapidly diminishes GFP fluorescence whereas excitation with a 470 nm light results in weak but stable green fluorescence[1]. If the wtGFP expressed in mammalian cells displays the same excitation and emission spectra as in vitro, then use of a conventional fluorescence microscope or a fluorescence activated cell sorter (FACS) will not provide optimal excitation wavelengths for the wtGFP to achieve maximal emission signals. Fluorescence microscopes typically contain a bandpass filter transmitting a blue light (~480 nm) which is required to excite fluorescein and its derivatives to emit a green light (~530 nm). Common flow cytometers contain an Argon ion laser tuned at 488 nm, and at 350 nm (UV light) if a second laser is added. In both cases, the wavelengths of exciting light sources, around 488 nm or 350 nm, are significantly different from the GFP's 395 nm excitation maximum, and therefore are not optimal for detecting the wtGFP fluorescence signal.

Two GFP mutants with excitation maxima around 490 nm have been created. One such red-shifted mutant was created by replacing 3 amino acids[8]. This mutant GFP (RSGFP4) exhibits excitation and emission peaks at 490 nm and 505 nm, respectively. The other GFP mutant has a single amino acid substitution of serine-65 to threonine, GFPS65T[9]. The *E. coli*-expressed GFPS65T displays excitation and emission maxima at 490 nm and 511 nm, respectively, and is sixfold brighter than the wtGFP[9].

We show that the two mutant GFPs display a much brighter green fluorescence than the wtGFP in living mammalian cells. When examined by FACS, GFPS65T and RSGFP4 are 18-fold and 24-fold, respectively, brighter than the wtGFP. In addition, we have created retroviral vectors containing the improved GFP gene and used these vectors to express GFP in transduced cells.

Results

Analysis of wildtype and mutant GFP protein levels in transfected mammalian cells. To compare the wildtype and mutant GFP gene expression in mammalian cells, expression vectors containing either the wtGFP, RSGFP4, or GFPS65T were transfected into BOSC23 fibroblasts in which the transgene can be highly expressed. After two days, monolayers of transfected cells were examined by fluorescence microscopy with a fluorescein filter set (see Experimental protocol). Cells transfected with either of the two mutant GFP genes displayed much brighter green fluorescence than cells transfected with the wtGFP (data not shown). To quantitate the relative fluorescence intensities of these three GFP variants, transfected cells were analyzed by a flow cytometer. The GFPS65T and RSGFP4 expressed in BOSC23 cells are approximately 18-fold and 24-fold, respectively, brighter than the wtGFP, provided the expression levels of all the three GFPs are similar (Fig. 1). Similar results were obtained with COS-7 cells although a smaller fraction of COS-7 cells was transfected (data not shown). In addition, bright green fluorescent signals from the expressed GFPS65T and

RSGFP4 proteins (recorded in the fluorescein channel) did not spill over into the second emission channel, which is used to record other fluorophores with an emission wavelength longer than fluorescein (data not shown). This allows us to analyze or sort cells coexpressing GFP and another marker, using an appropriate fluorophore which fluoresces in the second channel. Likewise, we can analyze cells using a fluorescence microscope equipped with a dual bandpass filter set for two color analyses.

Since the emission maximum of RSGFP4 is measured at 505 nm in vitro, we investigated whether a 515/20 nm filter (which selectively transmits 505 to 525 nm emission light) would be better than the standard filter (530/30 nm), which is optimal for fluorescein emission. Using the RSGFP4-transfected BOSC23 cells, we observed little difference in signals between the two filters. However, the background green fluorescence displayed in mock-transfected cells was twofold to threefold lower when the 515/20 nm filter was used (data not shown). Similar observations were also made with other cultured cells such as PA317 murine fibroblasts. Therefore, the ratio of the RSGFP4 signal to noise (i.e., background cell green fluorescence) in a FACS analysis can be improved twofold to threefold if a 515/20 nm emission filter is used.

GFP expression via retroviral-mediated gene transduction. An improved GFP gene that can be detected rapidly and noninvasively would be an invaluable tool for defining gene transduction strategies. Since retroviral vectors are widely used for stable gene transfer, the RSGFP4 gene was inserted into the murine stem cell virus vector (MSCV) to examine the use of GFP as a reporter gene for retroviral-mediated gene transfer and expression. In this GFP-expressing vector (MGPN), expression of the RSGFP4 gene is controlled by the long-terminal repeat (LTR) while a selection gene (neomycin) is driven by an internal promoter (Fig. 2). The MGPN vector was transfected into the BOSC23 ecotropic packaging cell line, and cells were analyzed by FACS two days after transfection. Approximately 28% of the cells emitted a green fluorescent signal (Fig. 3B). Supernatant from the transfected producer cells was then collected and tested for viral production measured by neomycin gene transduction of NIH3T3 cells. The end-point titer of the MGPN and MSCV retroviruses was similar, ranging from approximately 0.5 to 1×10^6 G418-resistant colony forming units per milliliter (cfu/ml). These results indicate that the RSGPP4 gene is not detrimental to virus production.

In addition to measuring retroviral transduction of NIH3T3 cells by neomycin selection, we also examined GFP expression mediated by infection with the MGPN retrovirus. NIH3T3 cells as well as an amphotropic packaging line, PA317 murine fibroblasts, were infected with either MGPN or the control MSCV virus. The infected cells were then cultured for four days in the absence of G418 selection, before living cells were harvested and analyzed by FACS. More than 90% of MGPN-infected PA317 cells emitted green fluorescence compared to 0.5% for control MSCV-infected cells (Fig. 4). MGPN-infected cells yielded a positive population with a peak fluorescence 15-fold greater than background fluorescence of the PA317 packaging cells. Therefore, GFP can be stably expressed in mammalian cells via a retroviral-mediated gene transduction. The GFP fluorescent signal in transduced NIH3T3 cells is less significant, mainly due to the high background fluorescence of NIH3T3 cells (data not shown).

To determine whether viable GFP-expressing cells could be selected by FACS based on their fluorescent signal, we sorted out and subsequently expanded the top 10% of the MGPN-infected PA317 cells displaying the brightest fluorescence. More than 90% of sorted cells were viable and resistant to G418. After 7 days expansion in culture, sorted producer cells displayed a brighter (approximately fourfold) fluorescent signal than the unsorted cells (Fig. 5). In addition, the end-point virus titer, produced by the sorted cells (approximately 1.0×10^6 cfu/ml), increased fourfold over unsorted PA.MGPN producer (0.2×10^6 cfu/ml).

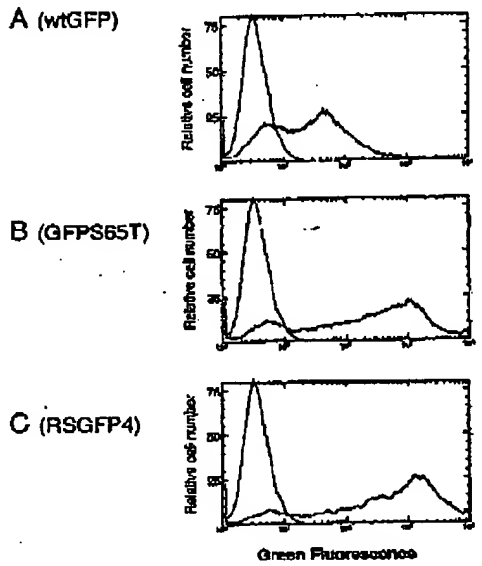

Figure 1. FACS analysis of GFP gene expression in transfected human cells. GFP-expressing plasmids containing either the wtGFP gene (A), GFPS65T (B), or RSGFP4 (C) were used to transfect BOSC23 cells. Forty hours post transfection, adherent cells were harvested and green fluorescence from transfected cells was analyzed by a flow cytometer tuned at 488 nm. The relative numbers of viable cells were then plotted as the function of variable intensities of green fluorescence from individual cells. The profile of mock-transfected cells (dotted lines) is overlaid for comparison. The relative intensities of the peak green fluorescence from cells transfected with pGFP-C1, pGFPS65T-C1 and pRSGFP-C1 are respectively 16-fold, 318-fold and 400-fold, greater than that of mock-transfected cells. Therefore, GFPS65T and RSGFP4 expressed in BOSC23 cells are approximately 18-fold and 24-fold, respectively, brighter than the wtGFP.

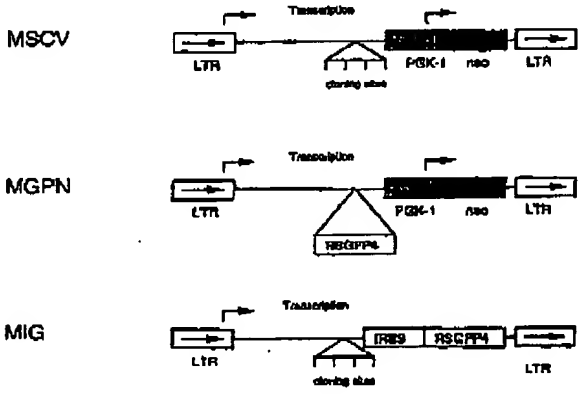

Figure 2. GFP-expressing retroviral vectors. Murine stem cell virus (MSCV) with a neomycin (neo) gene driven by an internal promoter PGK-1 is the parental vector. MGPN, the RSGFP4 gene is transcribed by the long-terminal repeat (LTR) of the MSCV. MIG, an internal ribosome entry sequence (IRES) was placed upstream of the RSGFP4 gene; the internal PGK-1 promoter and neo gene were deleted. See Experimental protocol for details.

To coexpress the GFP gene with another gene of interest, an

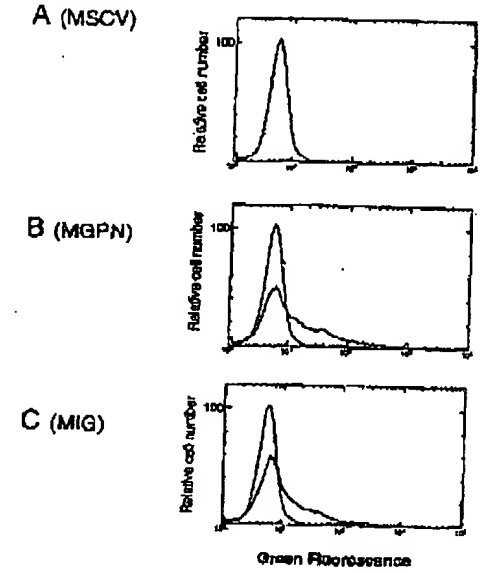

Figure 3. GFP expression in BOSC23 packaging cells transfected with retroviral vectors containing the RSGFP4 gene. BOSC23 cells were transfected with the MSCV (A), MGPN (B) or MIG (C) vector, and analyzed 2 days after transfection as in Figure 1. The profiles of cells transfected with MGPN (B) and MIG (C) are also overlaid with that of MSCV cells (dotted line). MGPN and MIG-transfected cells contain a cell population emitting green fluorescence (28% and 22.5% respectively) over the background (0.3% in MSCV-transfected cells).

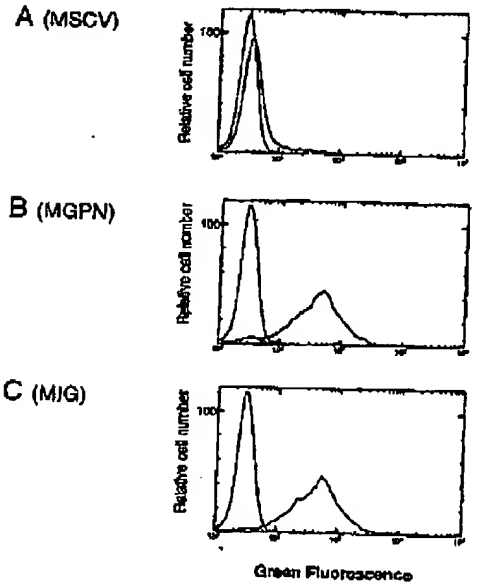

Figure 4. FACS analyses of GFP in transduced cells mediated by retroviral vectors. BOSC23 ecotropic packaging cells were transfected 3 days before the infection and viral-containing supernatants were subsequently collected. Then PA317 cells were then infected by either MSCV (A), MGPN (B) or MIG (C) viruses produced by BOSC23 producers, and analyzed 4 days after infection. The profile of mock-infected PA317 cells (dotted line) is overlaid. A 515/20 nm emission filter was used for the FL1 emission channel.

internal ribosome entry sequence (IRES) was placed upstream of the GFP gene in the MGPN vector after deleting the neomycin gene (Fig. 2). This construct creates two independent translational units, eliminating possible alterations in the GFP or the gene of interest resulting from the fusion of the two proteins. The IRES-containing GFP retroviral vector, MIG, displayed slightly weaker fluorescence in transfected BOSC23 packaging cells than the MGPN vector (Fig. 3C). MIG viruses produced by BOSC23 cells were then used to infect PA317 cells as before. The infected cells emitted a fluorescent signal similar to cells infected with the MGPN vector (Fig. 4C). Together these studies demonstrate that GFP variants such as RSGFP4 provide a simple method for selective cell sorting and isolation of high-titer viral producer clones based on the fluorescent signal. In addition, GFP variants can be coexpressed as a vital marker to monitor retroviral-mediated gene transfer and expression.

Discussion

We have analyzed the expression of wildtype and mutant GFP (GFPS65T and RSGFP4) in several types of mammalian cells and show that these proteins are stable and properly processed to form functional fluorophores. Expression of GFPs, either transiently or stably, are not detrimental to host cells. The GFPS65T and RSGFP4 are 18-fold and 24-fold, respectively, brighter than the wtGFP in mammalian cells as measured by flow cytometry. Therefore, these GFP variants are superior to the wtGFP for use with commonly used instruments optimized for fluorescein. In addition, the inclusion of RSGFP4 does not reduce the viral titer or the transduction efficiency of retroviral vectors. The improved GFP can be a useful marker either for monitoring gene transfer and expression, or for selecting transduced cells and high-titer viral producer clones.

A potential disadvantage of using GFP, over other reporter genes such as the lacZ gene, is its sensitivity limit. This could become critical in some applications in which the GFP gene can only be expressed at a low level. In these cases, GFP's green fluorescent signal may not be much over the background fluorescence of some target cells. Functional β-galactosidases (in tetrameric forms) encoded by the lacZ gene, in an hour can catalyze more than 10^6 substrate molecules, and prolonged incubation with excess substrates will further increase its maximal sensitivity[18]. However, the lacZ gene has some intrinsic limitations including: (1) endogenous β-galactosidase activities in some mammalian cells, (2) the requirements to transport fluorogenic substrates across cell membrane and to maintain the cleaved fluorescent products within viable cells, and (3) the enzymatic reaction time[19]. The latter two may limit the ability to sort viable cells expressing the lacZ gene, and prohibit real-time detection. In addition, the lacZ gene (3081 bp) is significantly larger than the GFP gene (710 bp), which may lead to reduced titers of retroviral vectors containing an additional gene of interest. Although we may not be able to reach the upper limit of sensitivity of the lacZ enzymatic assays, an improved GFP detection system and/or use of a further-improved GFP gene may constitute a more versatile reporter and vital marker that is sensitive enough for most biological applications.

We can envision several ways to further increase fluorescence sensitivity using GFP. One way is to place the improved GFP on the surface of cells to make it more accessible. Our preliminary experiments show that we can make a functional GFP associated with the outer cell membrane (data not shown). The localization of GFP on the cell membrane should also help the detection and imagery by fluorescence microscopy which is normally less sensitive than FACS. Alternatively, other GFP mutants known to be very bright in *E. coli*[11] may also be brighter in mammalian cells. Applications may include monitoring gene transfer and expression in gene therapy protocols, monitoring specific gene expression during critical developmental and disease states, and screening drugs which mod-

ulate the regulatory elements of gene expression.

Experimental protocol

DNA manipulations and expression vector constructions. Mammalian expression vectors containing either the wtGFP gene (pGFP-C1) or the RSGFP4 (pRSGFP-C1) were obtained from Clontech laboratories (Palo Alto, CA). In these vectors, the GFP genes are controlled by the immediate early promoter of human cytomegalovirus[4]. The vector containing the S65T point mutation was made by replacing the NdeI fragment (220 bp) in pGFP-C1 with a PCR-generated fragment containing a mutator primer based on the published sequence[2]. To make GFP-containing retroviral vectors, the 750 bp GFP fragment (from Eco47III to XhoI) from pRSGFP-C1 was cloned into the MSCVneoEB vector of the HpaI and XhoI sites[11]. The MSCVneoEB (MSCV) vector also contains a neomycin gene controlled by the internal PGK-1 promoter. This GFP-expressing retroviral vector is named MGPN. To create the retroviral vector MIG, in which the RSGFP4 is downstream to an internal ribosome entry sequence (IRES), the neomycin gene expression cassette (1300 bp) was first deleted from MGPN, and a 600 bp IRES fragment[12] was then inserted upstream to the RSGFP4 gene (the details of construction will be published elsewhere or provided upon request). All the plasmids were amplified in the DH5α *E. coli* strain (BRL, Gaithersburg, MD), and purified using a Promega's midi-preparation kit (Madison, WI).

Cell culture, transfection, fluorescence microscopy and FACS. Culture media (DMEM) were purchased from BRL and fetal calf serum (FCS) from Hyclone (Logan, UT). An ecotropic packaging cell line BOSC23, derived from the SV40 T antigen-transformed 293 human embryonic kidney (293T) fibroblasts, was cultured in DMEM plus 10% FCS[14]. The PA317 amphotropic packaging cells were cultured with DMEM plus 5% FCS[15]. For transfection, near-confluent BOSC23 cells were incubated with the precipitating mixture of $CaPO_4$ and saturating amounts of plasmids for 8 h before being replenished with fresh medium[14]. Unless otherwise indicated, BOSC23 cells were harvested by trypsin/EDTA (BRL) 40 h posttransfection, and resuspended in PBS plus 5 mM EDTA and 0.5% BSA for FACS analyses. Propidium iodide (0.5 μg/ml) was added to the cell suspension to exclude dead or dying cells from FACS analysis. Either a FACScan cytometer or FACStar^{plus} sorter (Becton Dickinson, San Jose, CA) equipped with an Argon ion laser tuned at 488 nm was used, and green fluorescence is recorded in the FL1 emission channel (normally used to detect fluorescein or its derivatives). Unless otherwise indicated, a standard 530/30 nm filter is used for the FL1 emission channel. We have also compared a 515/20 emission filter with the 530/30 filter in the FACStar^{plus} sorter (all filters are made by Chroma Technology Corp., Brattleboro, VT). Cells infected by different GFP-containing vectors were analyzed similarly. An Olympus fluorescence microscope with a mercury arc lamp (100 Watt) and a fluorescein filter set (consisting of a 480/40 nm excitation filter and a 535/50 nm emission filter) was used to detect GFP in living cells, or fixed cells treated by 3.6% para-formaldehyde. Fluorescence in living cells cultured on plastic dishes (with full media) is readily detectable within 24 h after transfection with the GFP65T-or RSGFP4-expressing plasmid. The signal was further enhanced when the media was replaced by PBS.

Retroviral production and infection. The BOSC23 cells were also used to test the retroviral constructs and to produce ecotropic retroviruses[14]. Two days after transfection, confluent BOSC23 cells were incubated with a minimal volume of fresh medium at 32°C. Then supernatant containing virus was collected every 12 to 24 h over the next 72 h. The endpoint titers of viral stocks were measured based on the neomycin gene transduction in NIH3T3 cells (10 days in the presence of 1 mg/ml active G418 from BRL). Viral infection was done essentially the same as the published protocol[16]. Afterward cell pellets were resuspended in fresh medium and cultured for 2 to 5 days before being analyzed by FACS.

Note added in proof

Yang et al. also reported that by using fluorescence microscopy, they can detect green fluorescence from RSGFP4 and wildtype GFP in CHO-K1 mammalian cells transfected respectively with the pRSGFP-C1 and pGFP-C1 expression plasmids. Yang, T.-T., Kain, S.R., Kitts, P., Kondepudi, A., Yang, M.M., and Youvan, D.C. 1996. Dual color microscopic imagery of cells expressing the green fluorescent protein and a red-shifted variant. *Gene (in press)*.

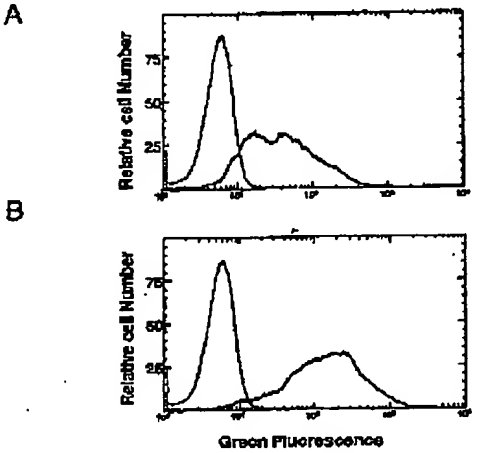

Figure 5. FACS analyses of GFP expression in stable PA317 packaging cells producing MGPN viruses. The PA317 packaging cells producing MGPN were generated by infection with BOSC23-produced MGPN viruses (see Fig. 4B). Part of these infected cells were used to establish a stable PA317 producer cell line (PA.MGPN) after G418 selection. The remainder were used to isolate the brightest 10% of GFP-expressing producers. After 7 days expansion in the presence of G418, both unsorted (A) and sorted (B) PA.MGPN producers were analyzed by FACS as described in Figure 1. The mean fluorescence intensity of sorted cells is fourfold greater than that of unsorted cells.

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09/710,058 Exhibit D

1: Biochem Biophys Res Commun. 1999 Jul 14;260(3):712-7. Related Articles, Links

Is green fluorescent protein toxic to the living cells?

Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ.

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Green fluorescent protein (GFP) has become more popular to be used as a living marker for positively transfected clones in many studies. To establish stable cell lines constitutively expressing GFP, three GFPs expressed from plasmid pBIEGFP, pSG5GFP, and pRSGFP were introduced into NIH/3T3, BHK-21, Huh-7, and HepG2 cells. All the GFPs we used are the mutant forms of a common wild phenotype. The pBIEGFP expressed enhanced GFP (EGFP). The pRSGFP and pSG5GFP expressed red shift GFP (RSGFP). The RSGFP gene in pSG5GFP was driven by a strong SV40 promoter and showed at least 20-fold higher RSGFP expression by western blot analysis. Despite of the variation in the levels of GFP expression, many GFP expressing cells contracted, rounded-up, and died, which was confirmed by decreasing luciferase activity. CPP32 activity and flow cytometric analyses further demonstrate that cells expressing GFP underwent apoptosis. Our observation is contradictory to other reports that GFP is nontoxic to the cells. Most importantly, this paper shows for the first time the link between expression of GFP and induction of apoptosis. This finding should promote studies of GFP cytotoxicity and attempts to isolate new non-toxic mutants of GFP. Copyright 1999 Academic Press.

PMID: 10403831 [PubMed - indexed for MEDLINE]

09/710,058 Exhibit E

1: Mol Ther. 2002 Oct;6(4):446-54.

Five recombinant simian immunodeficiency virus pseudotypes lead to exclusive transduction of retinal pigmented epithelium in rat.

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The purpose of our study was to evaluate lentiviral vector-mediated rat retina transduction using simian immunodeficiency virus (SIV) pseudotyped with envelope proteins from vesicular stomatitis virus G glycoprotein (VSV-G), Mokola virus G protein (MK-G), amphotropic murine leukemia virus envelope (4070A-Env), influenza A virus hemagglutinin (HA), lymphocytic choriomeningitis virus G protein (LCMV-G), and RD114 retrovirus envelope (RD114-Env). The six pseudotyped lentivirus vectors carried CMV-driven green fluorescent protein (GFP) or beta-galactosidase (beta-gal) reporter genes. Intravitreal and subretinal injections of each pseudotyped recombinant SIV were performed in cohorts of Wistar rats. Our results showed that no transgene expression was detected after intravitreal injection of each pseudotyped SIV vector. Also, no transduction could be detected following subretinal injection of RD114 pseudotyped SIV vectors. However, selective transduction of retinal pigment epithelium (RPE) cells was repeatedly obtained after subretinal delivery of VSV-G, MK-G, 4070A-Env, HA, and LCMV-G pseudotyped SIV. GFP expression was maximum as soon as 4 day postadministration for VSV-G, MK-G, 4070A-Env, and HA pseudotypes, with no evidence of pseudotransduction for VSV-G. Maximum transgene expression was observed 3 weeks postinjection for LCMV-6. Importantly, HA and VSV-G pseudotyped SIV lead to such a high level of transgene expression that GFP-related toxicity occurred. Therefore, when a high level of GFP synthesis is achieved, replacement of enhanced GFP (egfp, Acquorea victoria) by a low-toxicity GFP (Renilla reniformis) cDNA is necessary to allow long-term expression.

PMID: 12377185 [PubMed - indexed for MEDLINE]

09/710,058 Exhibit F-part 2

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